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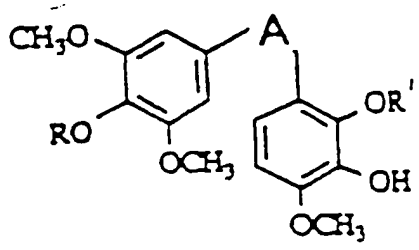

(I)

(57) Abstract

Herein disclosed are combretastatin derivatives (I) with antitumoral activity and a process for the extraction and isolation thereof; from *Combretum kraussii*; pharmaceutical compositions for antitumoral use and the preparation of said pharmaceutical compositions are also described, wherein R is H or CH_3, R' is H or β-D-glucopyranose, and wherein A is a bivalent radical having two carbon atoms selected from $-CH_2-CH_2-$ and $-CH=CH-$, provided that when R is CH_3 R' is different from H.

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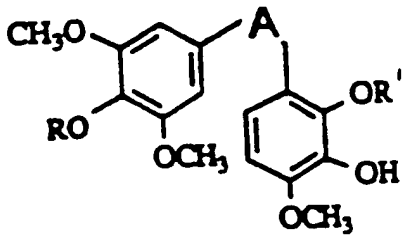
COMBRETASTATIN DERIVATIVES WITH ANTITUMORAL ACTIVITY AND PROCESS FOR THE PREPARATION THEREOF

1. Field of the invention

The present invention relates to combretastatin derivatives, with antitumoral activity, represented by the general formula (I):



wherein R is H or CH_3, R' is H or β-D-glucopyranose, and wherein A is a bivalent radical having two carbon atoms selected from $-CH_2-CH_2-$ and -CH=CH-, provided that when R is CH_3 R' is different from H;

and their pharmaceutically acceptable salts.

Combretastatin derivatives according to the invention are preferably prepared by extraction from seeds of Combretum kraussii according to a process which represents a further basic aspect of the present invention.

The derivatives according to the invention show a marked antitumoral activity and therefore they are particularly useful as active principle for the preparation of pharmaceutical compositions useful for treating tumors.

2. State of the art

Combretum species (Combretaceae) are widespread in tropical and subtropical areas where they find extensive use in indigenous medicine.

Different compounds have been obtained from Combretum genus, in particular triterpenoids from C. molle, C. padoides, e C. eleagnoides, phenanthrenes from C. hereroense, C. psidioides, C. apiculatum, e C. caffrum, amino acids from C. zeyheri.

In recent years two combretastatins, named A1 and B1 (Pettit et al., Journal of Natural Products, vol.50, No.1, 119-131, 1987), showing an in vitro antitumoral activity were isolated from C. caffrum,. Said A1 and B1 combretastatins inhibit the cell growth in vitro. They further inhibit the tubulin polymerization and consequently the microtubule assembly.

Chemical Abstract, vol.115, 1991, 189740y discloses A-4 combretastatin, isolated from C. caffrum, as a compound with antitumoral activity. Said combretastatin is an inhibitor of tubulin polymerization. The molecular structure of said combretastatin was determined by spectral techniques and confirmed by synthesis.

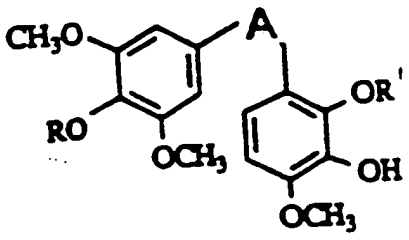
Technical problem

The continuous need of availability of new pharmaceutical products for antitumoral theraphy, draws toward the research and individualization of always more substances useful for treating tumors.

Detailed description of the invention

The invention refers to combretastatin derivatives with antitumoral

activity represented by the general formula (I)



wherein R is H or CH_3, R' is H or β-D-glucopyranose, and wherein A is a bivalent radical having two carbon atoms selected from $-CH_2-CH_2-$ and -CH=CH-, provided that when R is CH_3 R' is different from H;

and their pharmaceutically acceptable salts.

For easiness of exposition, in the present text are indicated as compound 1, 2, 3, 4, 5 and 6 the compounds of general formula (I) wherein the substituents are respectively:

1) R = CH_3, R'= H, A = -CH=CH- ;

2) R = CH_3, R'= H, A = $-CH_2-CH_2-$;

3) R = H, R'= H, A = $-CH_2-CH_2-$;

4) R = CH_3, R'= β-D-glucopyranose, A = -CH=CH- ;

5) R = CH_3, R'= β-D-glucopyranose, A = $-CH_2-CH_2-$;

6) R = H, R'= β-D-glucopyranose, A = $-CH_2-CH_2-$.

Compounds 1, 2 and 3 are also named respectively combretastatins A1, B1 and B5. Compounds 4, 5 and 6 are the 2'-O-β-D-glucopyranoside respectively of combretastatins A1, B1 and B5.

Compounds 3, 4, 5 and 6 are not disclosed in literature whilst

compounds 1 and 2 are known.

Glucopyranosides of combretastatins are easily soluble in aqueous solutions and therefore they are particularly useful for administration.

The antitumoral activity of glucopyranosides of combretastatins is not disclosed in literature.

A further basic characteristic of the invention is directed to the process of extraction and isolation, from C. kraussii, of the foregoing glucopyranosides of combretastatins A1, B1 and B5 and of the new combretastatin B5.

Another further characteristic of the invention is directed to the use, as active principle for the preparation of compositions with antitumoral activity, of at least a compound selected from 2'-O-β-D-glucopyranosides of A1, B1 and B5 combretastatins and the new B5 combretastatin.

A further characteristic of the invention is directed to pharmaceutical compositions with antitumoral activity, comprising as active principle at least a compound selected from the 2'-O-β-D-glucopyranosides of combretastatins A1, B1 and B5 and the new combretastatin B5.

The extraction and isolation of combretastatins and glucoside derivatives thereof from seeds of Combretum kraussii can be preferably performed by extraction and fractionation techniques with solvents and chromatographic columns.

According to a preferred embodiment of the invention the extraction

and isolation process of the desired compounds comprise the following steps:

a) seeds of C. kraussii are extracted with solvents having increasing polarity at a temperature comprised from room temperature and the boiling point of the used solvent for a time of from 2 to 10 days;

b) compounds extracted with low polarity solvents are collected by at low pression evaporation of the solvent at a temperature not higher than 60°C and then subjected to purification by flash chromatography on silica gel, eluting with binary mixture of hydrocarbons and ethyl acetate, or chlorinated solvents, ratio from 10:3 to 3:10 and performing more times the process until to obtain the desired purity degree;

c) compounds extracted with high polarity solvents are collected by at low pression evaporation of the solvent at a temperature not higher than 60°C, treated for 3 or more times with a mixture H_2O/n-butanol to set free the glucoside derivatives of combretastatins from by-products (sugars and other) more soluble in H_2O and the butanolic fraction is subjected to purification by flash chromatography on silica gel eluting with a mixture of medium polarity solvents and by furtherly purifying the upgraded fractions obtaining the desired compounds by reversed phase (RP) chromatography utilizing solvents with high polarities, alone or mixture thereof;

d) the purified fractions comprising the products of interest obtained from steps b) or c) are warmed at low pression at a

temperature not higher than 60°C until a constant weight in order to isolate the desired compound.

Preferred low polarity solvents for the process according to the invention are , aliphatic and cycloalyphatic hydrocarbons saturated and insaturated, petrol ether; preferred medium polarity solvents are aliphatic esters, in particular ethyl acetate (EtOAc) and clorinated hydrocarbons; preferred high polarity solvents are low molecular weight alcohols, aliphatic ketons, acetonitrile and H_2O.

According to a preferred embodiment of the process according to the invention step c) can be performed utilizing silica gel modified with chains preferably hydrocarbonic and a mixture of clorinated hydrocarbons or EtOAc and alcoholic solvents in ratio from 20:1 to 5:1.

n-butanolic phase shows bioactivity in the lethality test according to Meyer B.N. et al., Planta Medica, 45, 31-34, 1982, whilst the aqueous residue does not show bioactivity.

According to a further preferred embodiment of the invention the extraction of step b) is performed with a mixture of n-hexane/EtOAc 7:3.

The obtained extract shows bioactivity to the lethality test according to Meyer et al.

A further basic characteristic of the present invention is represented by a pharmaceutical composition with antitumoral activity comprising as active principle an effective amount of at least one of the compounds of general formula (I) wherein R is H or

CH_3, R' is H or β-D-glucopyranose, and wherein A is a bivalent radical having two carbon atoms selected from $-CH_2-CH_2-$ and -CH=CH-, provided that when R is CH_3 R' is different from H, or a pharmaceutically acceptable salt thereof, in combination with pharmaceutically acceptable suitable excipients and diluents.

Conventional excipients and diluents can be used and the compositions may be formulated in conventional manner.

Particularly preferred are pharmaceutical compositions with antitumoral activity wherein the active principle is constituted by an effective amount of at least one of the compounds of general formula (I) wherein R is H or CH_3, R' is 2'-O-β-D-glucopyranose, and wherein A is a bivalent radical having two carbon atoms selected from $-CH_2-CH_2-$ and -CH=CH-.

Data obtained from cell growth inhibition tests in vitro and from their comparison with data of other combretastatins (agluconates) known to be useful as antitumoral, permit of defining the 2'-O-β-D-glucopyranosides of combretastatins A1, B1 and B5, by considering their solubility in H_2O and therefore their extremely easiness of administration, as particularly useful in the antitumoral treatment. Said glucopyranosides of combretastatins probably show a mechanism of action different fron that of the agluconate combretastatins.

Example 1

a) Seeds of C. kraussii were collected in the National Botanic Garden, Pretoria, Transvaal.

Roughly minced seeds (155 g) were fed into a glass column. The column was then filled with 3.2 litres of petrol ether. The

extraction was carried out within 6 days. The collected extract was dried giving an amount of 3.04 g.

About 20 mg of the fraction extracted with petrol ether were employed to test the bioactivity by the "brine shrimp" (of Artemia salina) lethality test according to Meyer et al. It was given a value of LC_{50} (50% lethality concentration) of 269 ppm.

The fraction extracted with petrol ether, excepted for the little amount employed for the foregoing lethality test, was fractioned by flash chromatography, by using a column (15 cm in length, 5 cm diameter) fed with silica gel (Si gel 60®, 230-400 mesh) and eluted with 3 litres of a mixture of n-hexane/ethyl acetate (n-hexane/EtOAc) 7:3. 100 fractions of 30 ml each were collected, said fractions having been analyzed by TLC (Si gel plates, 5 x 10, 0.25 mm), by using n-hexane/EtOAc 7:3 as eluent. The collected fractions were tested and selected according to their composition:

i) Fractions numbered from 73 to 89 (30 ml each) were assembled and the amount was equivalent to 160 mg. Said amount was further purified by flash chromatography, on Si gel, by using a column (15 cm in length, 2 cm diameter) eluted with 1.2 litres of n-hexane/EtOAc 7:3. 120 fractions (10 ml each) were collected. Said fractions (10 ml each) were analyzed by TLC, and assebled according to their composition. Said fractions were dried, and from them respectively 7 mg of combretastatin A1 (compound 1) and 100 mg of combretastatin B1 (compound 2) (respectively 0.45×10^{-2}% and 6.45×10^{-2}% of dried material) were obtained.

ii) Fractions numbered from 90 to 100 (30 ml each) were assembled and the amount was equivalent to 66 mg. Said amount was furtherly purified by reverse phase medium pressure chromatography (RPMPLC) on Lichrosorb® RP 18 (diameter of the particles equal to 40-63 µm), by using a Buchi® 681 pump and a Buchi® 685 glass column at a flow rate of 1 ml/min. (in this case a column of 36 cm in length and 2 cm of diameter), and eluted with 200 ml of MeOH/H_2O 3:2. 100 fractions (2 ml each) were collected, said fractions were analyzed by TLC on RP 18, by using MeOH/H_2O 3:2 as eluent, then dried obtaining an amount of 40 mg (2.58×10^{-2}% of the dried material) of the compound 3 (a new compound named combretastatin B5).

Combretastatins A1 and B1 were confirmed according to the spectra data disclosed by Pettit et al.

Compound 3 was characterized, and the data are as follows:

HR (high resolution) EIMS (elctronic impact mass spectrometry) m/z (mass/electron) 320.3460 (71%, M^+, the value calcolated for $C_{17}H_{20}O_6$ is 320.3454), 167(90%), 153(100%). EI spectra were recorded on a VG 7070® mass spectrometer. ^1H NMR (200 MHz, $CDCl_3$) δ: 6.58 (1H, d, J=8.2 Hz, H-6'), 6.43(2H, s, H-2 + H-6), 6.39 (1H, d, J=8.2 Hz, H-5'), 5.45 (3H, bs, OH), 3.87 (9H, s, $-OCH_3$), 2.87 (4H, m, $-CH_2CH_2$). ^{13}C NMR value are reported in table 1.

b) The seeds previously subjected to the extraction with petrol ether were left out to dry in the air for 12 h, and then subjected to an extraction with methanol. The foregoing column was filled with 3.7 litres of methanol. The extraction was carried out within 6 days. The collected extract was dried giving an amount of 13.07 g.

Said 13.07 g were partitioned between a mixture of H_2O and n-butanol (100 ml of H_2O and 200 ml of n-butanol). The n-butanolic phase was dried to a weight of 3.1 g.

Little amounts of fractions extracted with n-butanol and aqueous residue were tested for bioactivity with the test of Meyer et al., giving a LC_{50} value of 67 ppm for the fraction extracted with n-butanol, whilst the aqueous residue did not give significant values.

The 3.1 g of the fraction extracted with n-butanol, excepted for the little amount (about 20 mg) used for the lethality test, were pre-purified by flash chromatography on Si gel, by using a column 15 cm in length and 5 cm of diameter, and elueted with 2.1 litres of $CHCl_3$/MeOH 9:1.

70 fractions (30 ml each) were collected, said fractions were monitored by TLC (by using $CHCl_3$/MeOH 9:1 as eluent for TLC), then assembled according to their composition:

i) The fractions numbered from 26 to 42 (30 ml each) were assembled for an amount of 313 mg. Said amount was furtherly purified by RPMPLC on Lichrosorb® RP 18 (diameter of the particles equal to 40-63 μ), by using a column of 50 cm in length and 2 cm in diameter, then eluted with 300 ml of MeOH/H_2O 3:2 giving 150 fractions (2 ml each).

The fractions numbered from 97 to 121 (2 ml each) were assembled together and dried; 65 mg (4.19×10^{-2}% of the dried material) of the compound 5 were obtained.

The fractions numbered from 122 to 146 (2 ml each) were assembled

together for an amount of 156 mg. Said amount was furtherly purified, firstly by RPMPLC on RP 18, by using a column of 50 cm in length and 2 cm of diameter, and then on RP 8 (diameter of the particles equal to 25-40 μm), by using a column of 50 cm in length and 2 cm of diameter, then eluting with 75 ml of MeOH/H_2O 55:45, obtaining 50 fractions (1.5 ml each). Such fractions (1.5 ml each) comprising the product of interest were assembled and dried giving 6 mg of the compound 4 (o.39 x 10^{-2}% of the dried material).

ii) The fractions numbered from 43 to 70 (30 ml each) were assembled giving an amount of 110 mg. Said amount was purified by RPMPLC on RP 18, by using a column of 50 cm in length and 2 cm of diameter, then eluting with 150 ml of MeOH/H_2O 3:2, obtaining 100 fractions (1.5 ml each). The fractions comprising the product of interest were assembled, then dried, giving 25 mg of the compound 6 (1.61 x 10^{-2}% of the dried material).

The compound 4 (6 mg, 0.39 x 10^{-2}%) was characterized and the data are as follows: the mass spectra were recorded on VG 7070® FAB (fast atomic bombardment) MS (mass spectroscopy)(negative) m/z 493 $(M-H)^-$. ^1H NMR (200 MHz, DMSO d_6) δ: 6.87 (1H, d, J=12 Hz, -CH=CH-), 6.66 (1H, d, J=7.8 Hz, H-6'), 6.58 (1H, d, J=7.8 Hz, H-2'), 6.50 (2H, s, H-2 + H-6), 6.41 (1H, d, J=12 Hz, -CH=CH-), 5.23, 5.05 (bs, OH), 4.57 (1H, d, J=8 Hz, H1''), 3.71 (3H, s, $-OCH_3$), 3.62 (3H, s, $-OCH_3$), 3.55 (6H, s, $-OCH_3$). ^{13}C NMR values are reported in table 2. The 65 mg of the compound 5 were treated with EtOAc giving an amorphous powder.

As regard other tests of activity, the compound 5 was purified by

HPLC until a title of 92%. The remaining part being essentially constituted of the 2'-O-β-D-glucopyranosides of combretastatins A1 and B5. The solubility in H_2O of such compound 5 is 0.5 mg/µm.

The values regarding the characterization of compound 5, with reference to ^{13}C NMR values, are reported in table 2, whilst the ^{1}H NMR values are as follows:

Compound 5 was precipitated with EtOAc as amorphous powder. p.dec. (decomposition point) 60°C;$[\alpha]^{25}_D$ + 2,2 (c 1, MeOH). ^{1}H NMR (200 MHz, DMSO d_6) δ:6.68 (1H,d, J=8.6 Hz, H-6'), 6.60 (1H, d=8.6 Hz, H-2'), 6.54 (2H, s, H-2 + H-6), 5.23, 5.15 (bs, OH) 4.52, (1H, d, J=7.5 Hz, H-1''), 3.74 (6H, s, $-OCH_3$), 3.72 (3H, s, $-OCH_3$), 3.61 (3H, s, $-OCH_3$).

The author of the present invention, through the acetylation and the conseguent variations obtained from ^{13}C NMR studies, have determined the binding position of glucose. The compound 5 was acetylated with known methods, obtaining the compound 5a: $[\alpha]^{25}_D$= -3,3 (c 0,4 , $CHCl_3$). EI MS m/z 706 $(M)^+$, 375 $(C_{20}H_{23}O_7)^+$, 331 $(C_{14}H_{19}O_9$, 100%$)^+$, 181 ($C_{10}H_{13}O_3)^+$. ^{1}H NMR (200 MHz, $CDCl_3$) δ: 6.93 (1H, d, J=8.8 Hz, H-6'), 6.71 (1H, d, J=8.8 Hz, H-2'), 6.38 (2H, s, H-2 + H-6), 5.38-5.12 (3H, m), 5.02 (1H, d, J=7.0 Hz, H-1''), 4.27 (1H, dd, J=12.5, 4 Hz, H-6a''), 4.02 (1H, dd, J=12.5, 2.5 Hz, H-6b''), 3.86 (6H, s, $-OCH_3$), 3.84 (3H, s, $-OCH_3$), 3.82 (3H, s, $-OCH_3$), 3.60 (1H, m, H-5''), 2.80 (4H, m, $-CH_2CH_2-$), 2.38 (3H, s, $\underline{CH_3}COOAr$), 2.06 , 2.04 , 2.02 , 1.92 (12H, s, $\underline{CH_3}COOR$).

^{1}H and ^{13}C NMR spectra were recorded on a Brucker® AC 200E

spectrometer, using TMS as internal standard. All the operations of the above process of extraction were performed at room temperature.

c) Hydrolisis of the compounds.

Hydrolisis of compounds 4, 5 and 6 were realized for a further verification, showing that said compounds are the correspective β-D-glucoryranosides of compounds 1, 2 and 3.

5-10 mg of each of the compounds 4, 5 and 6 were treated with 3% HCl/MeOH, for 3 h at 60°C, under N_2. The solvent was removed with a flow of N_2 and dried under vacuo. The mixtures were taken up with water and extracted with $CHCl_3$. The presence of glucose as methyl glucose was identified, by TLC (5 x 10 cm, 0.25 mm) and GLC, in the aqueous phases, whilst the aglucones, that is the compounds 1, 2 and 3, were indentified, by TLC and ^1H NMR, in the chlorophormic extract.

Compounds 1 and 2, according to their ^1H NMR values, correspon exactely to the combretastatins A1 and B1, previously isolated from C. caffrum. Also the ^1H NMR values of compound 1 and 2, recorded in $CDCl_3$ are in compliance with those of combretastatins A1 and B1 .

Compound 3, i.e. combretastatin B5, has a 4-hydroxy group instead of the 4-O-methoxy group of combretastatin B1, and it is a new combretastatin.

The data of ^1H NMR spectra for compounds 4, 5 and 6 show the signal of an anomeric proton at δ 4.52-4.57, and doublets with J equal about to 8 Hz. Such value is typical of a β linkage.

The values of the chemical shifts of ^{13}C of compounds 1-6 are reported in tables 1 and 2.

Tabella 1

^{13}C NMR (52.4 MHz) chemical shifts assignments of compounds 1, 2 and 3.

C	1 *	1 **	2 *	2 **	3 *	m
1	132,89	132,74	138,08	138,02	132,69	s
2	106,49	106,16	105,25	105,47	105,12	d
3	153,40	153,16	152,86	152,70	146,72	s
4	137,90	#	135,85	135,53	133,37	s
5	153,40	152,46	152,86	152,70	146,72	s
6	106,49	106,16	105,25	105,47	105,12	d
1a	130,88 d	128,04 d	36,60	36,20	36,36	t
1'a	124,65 d	125,89 d	31,77	31,59	31,93	t
1'	118,34	117,80	121,37	121,43	121,44	s
2'	142,15	144,40	142,00	143,96	142,02	s
3'	133,80	133,93	132,13	133,76	132,17	s
4'	146,91	147,93	145,21	146,81	145,20	s
5'	103,50	103,34	102,30	102,72	102,35	d
6'	120,94	119,08	120,00	119,01	120,06	d
4 OCH_3	61,50	60,14	60,77	60,04	-	q
4'OCH_3	56,46	55,62	56,04	55,84	56,05	q
3,5OCH_3	56,75	55,95	55,93	55,78	56,12	q

m= multeplicities as determined by DEPT experiment.

* values in $CDCl_3$;

** values in DMSO d_6;

this value is not clearly distinguishable

Tabella 2

^{13}C NMR (52.4 MHz, DMSO d_6) chemical shifts assignments of compounds 4, 5 and 6.

C	4	5	5a*	6	m
1	132,51	138,10	137,69	132,43	s
2	106,19	105,63	105,60	105,96	d
3	152,48	152,65	152,75	144,77	s
4	136,59	135,45	135,47	133,34	s
5	152,48	152,65	152,75	147,77	s
6	106,19	105,63	105,60	105,96	d
1a	128,90 d	36,91	36,22	36,58	t
1'a	126,66 d	31,18	31,40	31,35	t
1'	123,88	128,03	127,81	128,22	s
2'	143,80	143,93	147,70	143,95	s
3'	139,37	139,35	140,33	139,29	s
4'	148,15	146,95	150,36	146,90	s
5'	108,56	108,99	109,02	109,10	d
6'	118,90	118,56	126,60	118,68	d
4 OCH_3	60,15	60,04	60,05	-	q
4'OCH_3	55,96	55,94	55,80	56,00	q
3,5OCH_3	55,62	55,78	55,75	56,00	q
1''	105,83	105,77	99,91	105,79	d
2''	73,97	74,09	70,95	74,04	d
3''	76,28	76,26	71,45	76,14	d
4''	69,75	69,76	68,06	69,71	d
5''	77,46	77,51	71,96	77,44	d
6''	60,90	60,96	61,32	60,89	t

m= multeplicities as determined by DEPT experiment.

* The signals of acetyl groups are at δ 20.44 (5 x $COO\underline{CH_3}$), 169.95, 169.69, 169.31, 169.21, (R-O-$\underline{CO}CH_3$) e 167.50 (Ar-O-$\underline{CO}CH_3$).

The comparison between the values reported respectively for compounds 2 and 5, with reference to carbons C1', C3' and C5', shows a deshielding effect of 6-7 ppm in compound 5 compared with compound 2. Similar deshielding effects were observed in carbons C2', C4' and C6' when the compound 5 was acetylated, giving the 5a compound. This

data confirms that the compound 5 is the 2'-O-β-D-glucopyranoside of compound 2. Compounds 4 and 6 show the same ^{13}C NMR deshielding effects (described for compound 5) compared to their aglucones (respectively the compounds 1 and 3). From these considerations, it is confirmed that compound 4 is the 2'-O-β-D-glucopyranoside of combretastatin A1 and compound 6 is the 2'-O-β-D-glucopyranoside of combretastatin B5.

The obtained compounds were subjected to a water solubility test. The possibility that a compound could be efficiently used in theraphy is strongly increased if said compound is soluble in water and therefore easily formulable.

It was experimently found that the solubility in water of the glucosides of combretastatins is higher than that of their agluconate compounds.

Example 2

Compound 5, prepared as described in example 1, was utilized in tests in vitro, to verify its capacity in inhibiting the growth of mouse leukemia L1210 cells. Exponentially growing L1210 cells, were treated for 24 hours with compound 5 at 20 and 30 μM concentrations. It was observed a cell growth inhibition by 50% after 24 hours exposure at the concentration of 20 μM. Compound 5 was firstly dissolved as stock solution in undiluted DMSO at a 30 mM concentration and afterwards diluted in water as a 100-fold concentrate in respect to the final concentration (20 and 30 μM).

Cells culture preparation.

L1210 mouse lymphocytic leukemia cells (0.1×10^6 cells/ml) were

grown in suspension culture in RPMI 1640 medium (Gibco®), supplemented with 10% heat inactivated (56°C, for 30 min) fetal bovine serum (Gibco®) and 2-mercaptoethanol 10μM. Stock cultures were maintained in exponential growth at a density between 0.1×10^6 and 1×10^6 cells/ml. Cells were routinely found to be free of Mycoplasma contamination by staining with Hoechst® 33258 dye and examining under a fluorescent microscope.

Growth inhibition test in vitro.

L1210 cells were seeded at a concentration of 1×10^5/ml in Nunc T25 flasks (Gibco®). 24 hours later, cells were treated with compound 5 (at the concentration reported at point f) for 24 hours. At the end of the treatment, cultures were washed with prewarmed phoshate buffered solution (PBS) by centrifugation and resuspended in fresh drug-free medium. 24 hours after compound 5 exposure, and 24, 48 and 72 hours after compound 5 washout, cell growth inhibition was evaluated by counting treated and untreated (control) cells in a Coulter Counter (Coulter Electronics® Ltd.). Date are the average of four replications. Controls and treated samples were diluted in fresh medium after seeding in order to maintain he cell in logarithmic growth phase.

Example 3

Compound 5 prepared as described in example 1, was utilized in in vitro studies to evaluate its capacity of inhibiting the tubulin polymerization.

It is known that compounds as combretastatins A1, A4 and B1,

podophyllotoxin, steganacin and colchicine are significant potent inhibitors of tubulin polymerization, which is the major protein component of microtubules; said components, therefore, cause mitotic arrest in cells culture.

1.5 mg/ml of microtubule protein are polymerised, in vitro, in presence of various amounts of compound 5, showing a typical assemply kinetic with an initial lag-phase followed by a rapid elongation which tended to achieve a plateau by about 10 min. Increasing amounts of compound 5 did not have inhibitory effect on microtubule assembly showing the absence of the typical effect of inhibition of tubulin polymerization, characteristic of combretastatins.

The effective mechanism of antitumoral action of compound 5 is, until now, not understood. Further studies are in progress, by the authors of the present invention, to elucidate the mode of action of compound 5. However, it is possible to suppose, considering that compound 5 does not inhibit tubulin polymerization, that said compound 5 acts with a different mechanism of action from that of the known combretastatins.

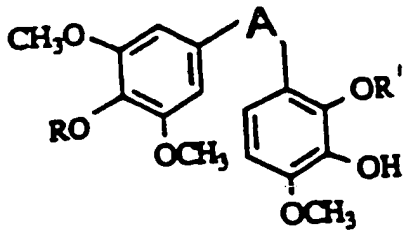
Tubulin, the major protein component of microtubules, was purified from whole bovin brain according to the method described by Burns R.G. and Islam K.(Annales of the New York Academy of Sciences, 466, 340-356, 1986) with some modifications. Brain was cooled to 4°C, homogenised in PIPES buffer (0.1 M PIPES, 2.5 mM EGTA, 0.5 mM $MgCl_2$, 0.1 mM EDTA, pH 6.9) and after centrifugation the supernatant was added with 20% glycerol, 1mM GTP and incubated for 20 min at 37°C.

After centrifugation the microtubule pellet was dissolved in MES buffer (0.1 M MES, 2.5 mM EGTA. 0.5 mM $MgCl_2$, 0.1 mM EDTA, pH 6.4). 13 mg/ml of the microtubule protein were stored in liquid nitrogen. Microtubule protein concentration was determined using a protein reagent according to Bradford M.M. (Analytical Biochemistry, 72, 248-254, 1976), and bovine serum albumin was used as standard. Assembly of twice cycled microtubule protein was initiated in MES buffer by the addition of GTP 0.5 mM, DTT 1mM and increasing the temperature at 37°C. Assembly kinetics were monitored at a wavelenght of 350 nm measuring the increase of light scattering according to Gaskin F. et al.(Journal of Molecular Biology, 89, 737-758, 1974).

The reported data of tubulin polymerization test show that compound 5 does not inhibit the tubulin polymerization, and therefore it presents a mechanism of action different from that of combretastatin B1.

Claims

1. Combretastatin derivatives with antitumoral activity of general formula (I):



wherein R is H or CH_3, R' is H or β-D-glucopyranose, and wherein A is a bivalent radical having two carbon atoms selected from $-CH_2-CH_2-$ and -CH=CH-, provided that when R is CH_3 R' is different from H:

and their pharmaceutically acceptable salts of addition.

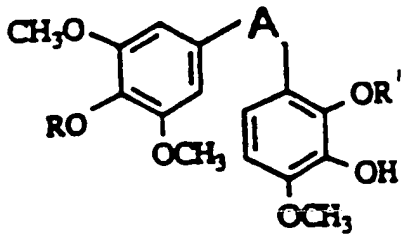
2. Combretastatin derivatives with antitumoral activity of general formula (I) according to claim 1, wherein R is CH_3, R' is β-D-glucopyranose, and wherein A is $-CH_2-CH_2-$.

3. Combretastatin derivatives of general formula (I) according to formula (I) wherein R is CH_3, R' is β-D-glucopyranose, and wherein A is -CH=CH-.

4. Combretastatin derivatives of general formula (I) according to claim 1, wherein R is H, R' is β-D-glucopyranose, and wherein A is $-CH_2-CH_2-$.

5. Combretastatin derivatives of general formula (I) according to claim 1, wherein R is H, R' is H, and wherein A is $-CH_2-CH_2-$.

6. Process for the extraction and isolation, from Combretum kraussii, of compounds of general formula (I)



wherein R is H or CH_3, R' is H or β-D-glucopyranose, and wherein A is a bivalent radical having two carbon atoms selected from $-CH_2-CH_2-$ and -CH=CH-, provided that when R is CH_3 R' is different from H,

comprising the following steps:

a) seeds of C. kraussii are extracted with increasing polarity solvents at a temperature comprised from room temperature and the boiling point of the used solvent for a time of from 2 to 10 days;

b) compounds extracted with low polarity solvents are collected by evaporation at low pression of the solvent at a temperature not higher than 60°C and then subjected to purification by flash chromatography on Si gel, eluting with binary mixture of hydrocarbons and ethyl acetate, or chlorinated solvents, in ratio from 10:3 to 3:10 and performing more times the process until to obtain the desired purity degree;

c) compounds extracted with high polarity solvents are collected by

evaporation at low pression of the solvent at a temperature not higher than 60°C, treated for 3 or more times with a mixture H_2O/n-butanol to set free the glucoside derivatives of combretastatins from by-products (sugars and other) more soluble in H_2O and the butanolic fraction is subjected to purification by flash chromatography on Si gel eluting with a mixture of medium polarity solvents and by furtherly purifying the upgraded fractions obtaining the desired compounds by reversed phase (RP) chromatography utilizing solvents with high properties, alone or a mixture thereof; d) the purified fractions comprising the products of interest obtained from steps b) or c) are warmed at low pression at a temperature not higher than 60°C until a constant weight in a way of isolating the desired compound.

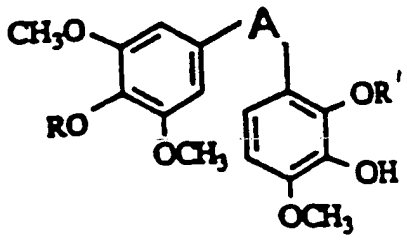
7. Process according to claim 6, characterized by the fact that the used solvents are selected from the group of aliphatic and cycloalyphatic hydrocarbons satured and insaturated, petrol ether, aliphatic esters, ethyl acetate and clorinated hydrocarbons, low molecular weight alcohols, aliphatic ketons, acetonitrile and H_2O.

8. Process according to claim 6, characterized by the fact that the flash chromatography of step c) is performed by silica gel modified with hydrocarbonic chains and a mixture of clorinated hydrocarbons or EtOAc and alcoholic solvents in ratio from 20:1 to 5:1.

9. Process according to claim 6, characterized by the fact that the extraction of step b) is performed with a n-hexane/ethyl acetate mixture in ratio 7:3.

10. Pharmaceutical composition with antitumoral activity comprising

as active principle an effective amount of at least one of the compounds of general formula (I)



wherein R is H or CH_3, R' is H or β-D-glucopyranose, and wherein A is a bivalent radical having two carbon atoms selected from $-CH_2-CH_2-$ and -CH=CH-, provided that when R is CH_3 R' is different from H, or a pharmaceutically acceptable salt thereof, in combination with pharmaceutically acceptable excipients and diluents.

11. Pharmaceutical composition according to claim 10, comprising a compound of formula (I) wherein R is H or CH_3, R' is β-D-glucopyranose, and wherein A is a bivalent radical having two carbon atoms selected from $-CH_2-CH_2-$ and -CH=CH-.

12. Pharmaceutical composition according to claim 11, comprising the compound of formula (I) wherein R is CH_3, R' is β-D-glucopyranose, and wherein A is $-CH_2-CH_2-$.

13. Pharmaceutical composition according to claim 11, comprising the compound of formula (I) wherein R is CH_3, R' is β-D-glucopyranose, and wherein A is -CH=CH-.

14. Pharmaceutical composition according to claim 11, comprising the compound of formula (I) wherein R is H, R' is β-D-glucopyranose, and wherein A is $-CH_2-CH_2-$.

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/EP 93/02173

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C07H15/203 C07C43/23 A61K31/70 A61K31/085

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C07H C07C A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP,A,0 276 051 (ARIZONA STATE UNIVERSITY) 27 July 1988 see claims 1-7 ---	1-14
Y	J. MED. CHEM. vol. 34 , 1991 pages 2579 - 2588 M. CUSHMAN ET AL. 'Synthesis and Evaluation of Stilbene and Dihydrostilbene Derivatives as Potential Anticancer Agents That Inhibit Tubulin Polymerisation' see tables I-V ---	1-14
Y	PATENT ABSTRACTS OF JAPAN vol. 010, no. 377 (C-392)16 December 1986 & JP,A,61 171 427 (OSAKA CHEM LAB) 2 August 1986 see abstract ---	1-5, 10-14
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 January 1994	27.01.94

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/EP 93/02173

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP,A,0 472 053 (EISAI) 26 February 1992 see claims 1,9; examples 73,74	1-5, 10-14
P,X	CHEMICAL ABSTRACTS, vol. 119, no. 21, 22 November 1993, Columbus, Ohio, US; abstract no. 221659z, F. PELIZZONI ET AL. 'Cell growth inhibitor constituents from Combretum krasussi' see abstract & NAT. PROD. LETT. vol. 1, no. 4 , 1993 pages 273 - 280	1-14
P,Y	WO,A,92 16486 (ASTON MOLECULES) 1 October 1992 see page 1, line 28 - page 3, line 36; claims 10,18	1-5, 10-14

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Int cnal Application No
PCT/EP 93/02173

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		US-A-	4996237	26-02-91
EP-A-0472053	26-02-92	AU-B-	636239	22-04-93
		JP-A-	5039256	19-02-93
		US-A-	5250549	05-10-93
		AU-A-	8249391	27-02-92
		CN-A-	1059519	18-03-92
WO-A-9216486	01-10-92	AU-A-	1371992	21-10-92